Neuron, Vol. 46, 521–528, May 19, 2005, Copyright ©2005 by Elsevier Inc.

An Unconventional Role of Neurotransmission in Synapse Formation

How do presynatic inputs regulate synapse formation? In this issue of *Neuron*, Lin et al. show that the neurotransmitter acetylcholine decreases the stability of AChR clusters. This dispersing activity, which requires the serine/threonine kinase Cdk5, cooperates with positive signals from motoneurons to ensure high concentration of AChRs at the neuromuscular junction.

The mammalian neuromuscular junction (NMJ) is a cholinergic synapse between motoneuron axons and skeletal muscles. Acetylcholine (ACh), released from the nerve terminals, activates ACh receptors (AChR) heavily concentrated in the postjunctional membrane. The ensuing sodium influx depolarizes the muscle cell, triggering calcium release from the sarcoplastic reticulum to initiate muscle contraction. Because of the easy access and peripheral location, the NMJ has been the most extensively studied model for synaptogenesis. NMJ formation requires coordinated interactions among motoneurons, muscle fibers, and Schwann cells. Past studies of neuromuscular synapses in culture suggest a dominant role for the neuron. Axons seem to form synapses at new locations, ignoring AChR-rich sites or hot spots preexisting on muscle fibers (Sanes and Lichtman, 1999). Moreover, several factors have been identified that appear to be utilized by motoneurons to govern the NMJ formation. For example, agrin induces AChR clusters, and elimination of the agrin gene or that of the downstream transmembrane receptor tyrosine kinase MuSK prevents NMJ formation (Sanes and Lichtman, 1999).

Recent studies of genetically manipulated mice, however, have revealed a nerve-independent mechanism for AChR clustering. Before the arrival of the nerve, AChR-rich sites form in the central region of the muscle fiber, outlining a broad band perpendicular to the long axis of muscle fibers (Lin et al., 2001; Yang et al., 2001). Unlike that of the adult NMJ, this band is wider and contains AChR-rich sites that are not apposed by the presynaptic nerve even after the arrival of nerve terminals in E14.5 mice. The innervation induces new AChR clusters or modulates existing AChR-rich sites beneath nerve terminals and concomitantly disperses those in nonsynaptic areas. Induction of AChR clustering is likely to be mediated by agrin. However, the dispersing signal remained elusive until the recent studies of mice deficient in choline acetyltransferase (ChAT), the biosynthesis enzyme for ACh. Unable to produce ACh, ChAT mutant mice lack nerve-evoked potentials in the muscle. Interestingly, AChR clusters appear to grow faster in ChAT mutant mice; the clusters are initially similar in size but are eventually 2- to 3-fold larger than those in age-matched controls (Brandon et al., 2003; Misgeld et al., 2002). These results suggest that nerves may use ACh to disassemble or refine AChR clusters. However, interpretation of the phenotypes in ChAT mutant mice was complicated by the expression of agrin, which promotes AChR clustering.

In this issue of *Neuron*, Lee, Lin, and colleagues (Lin et al., 2005) provide convincing evidence that establishes ACh as a dispersing signal and suggest mechanisms for how it scatters AChR-rich sites in nonsynaptic areas. They first compared the phenotypes of single agrin knockout mice with those in agrin/ChAT double mutants and found that more AChR clusters are present at E17.5 in the double mutants than those in single knockout mice. AChR clusters are larger and are distributed in a broader central region. By eliminating the endogenous agrin, these experiments demonstrated nicely that ACh may be a signal utilized by motoneurons to scatter AChR-rich sites and reduce the size of individual AChR clusters. In support of this notion are the observations that treatment of myotubes with ACh causes a decrease in AChR clusters formed spontaneously or induced by agrin (Lin et al., 2005; Sanes and Lichtman, 1999). Through a series of elegant experiments, Lin et al. went on to demonstrate that Cdk5, a cytoplasmic serine/threonine kinase (Cruz and Tsai, 2004), plays an important role in AChR cluster dispersal. First, ACh activates Cdk5 in cultured myotubes. Second, agonist-induced dispersion of AChR clusters is blocked in myotubes treated with the Cdk5 inhibitor roscovitine or in Cdk5 mutant muscle cells. To investigate the Cdk5 involvement in vivo, the authors injected roscovitine into pregnant mice and studied the effect of Cdk5 inhibition on NMJ formation in utero. AChR-rich sites persist in E17.5 single agrin mutant mice treated with roscovitine. In agreement, more AChR-rich sites and clusters (apposed to nerve terminals) are present in Cdk5/agrin double mutant mice than in single agrin mutant mice. Because Cdk5 has been implicated in regulating presynaptic activity (Cheng and Ip, 2003; Cruz and Tsai, 2004), the in vivo phenotypes may result indirectly from impaired ACh release. Nevertheless, these results, together with in vitro studies, make a compelling case that ACh serves as a signal to disperse preexisting AChR-rich sites in nonsynaptic areas and refine AChR clusters at the synapse in a manner dependent on the cytoplasmic kinase Cdk5.

The findings by Lin et al. inspire a number of interesting questions about the formation and the maturation of the NMJ as well as synaptogenesis in the brain. ACh can be released from approaching axons and stimulate the AChR immediately upon contact (Sanes and Lichtman, 1999). How could AChR clusters form and maintain at synapses when the negative signal ACh is constantly present? The authors addressed this question in cultured muscle cells. They showed that ACh dispersal of AChR clusters is diminished by agrin, indicating that agrin antagonizes the dispersion (Lin et al., 2005). These results can be explained by a model de-

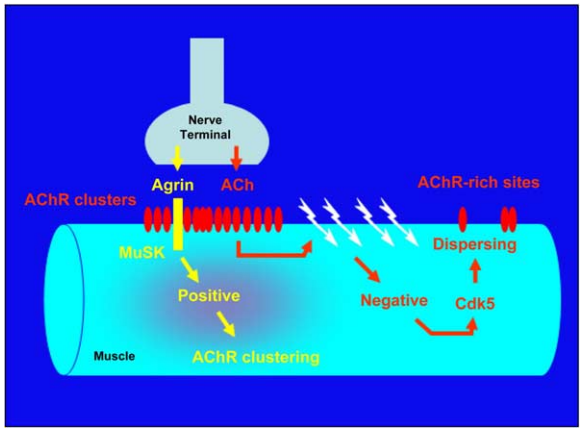

Figure 1. A Model for AChR Concentration at the NMJ

Nerves control AChR clustering by both positive and negative signals. ACh may serve as a negative signal, which activates AChR and subsequently Cdk5, to disperse AChR-rich sites in nonsynaptic areas and to modulate AChR clusters in the synapse region. AChR aggregation at synapses is induced and maintained by a signaling gradient generated by positive signals such as neural agrin. See text for details.

picted in Figure 1. The dispersing activity of ACh propagates globally to scatter AChR-rich sites or prevent their formation in the entire muscle fiber. Synaptic AChR clusters are induced and maintained by a signaling gradient generated by local activation of the agrin/MuSK machinery. Consistent with this idea is the enrichment of agrin, MuSK, and downstream signaling molecules at the NMJ (Finn et al., 2003; Luo et al., 2002; Sanes and Lichtman, 1999).

Is ACh the only signal from nerves to disperse AChR-rich sites? The answer seems to be no, because aneural diaphragms in HB9 mutant mice (in which the phrenic nerve fails to form) form more AChR-rich sites than in agrin/ChAT double knockouts (Lin et al., 2005). Interestingly, neuregulin was recently shown to inhibit AChR aggregation in myotubes (Trinidad and Cohen, 2004). This factor is believed to be utilized by motoneurons to mediate synapse-specific transcription and can activate Cdk5 in muscle cells (Fischbach and Rosen, 1997; Fu et al., 2001; Schaeffer et al., 2001). Cdk5 activation has been proposed to regulate AChR clustering (Cheng and Ip, 2003). However, it remains to be tested whether neuregulin has such a role in vivo. It is unlikely that neuregulin serves as a signal to disperse AChR clusters far away from synapses, because neuregulin and its receptor ErbB tyrosine kinases are localized at the NMJ (Fischbach and Rosen, 1997). The failure of ACh agonists to disperse AChR clusters in muscle cells when Cdk5 is inhibited or deficient suggests that Cdk5 is a key effector for AChR cluster dispersal. This notion predicts similar NMJ phenotypes in ChAT and Cdk5 mutant mice. However, while AChR cluster sizes in ChAT mutant mice are bigger than in age-matched controls, those in Cdk5 mutant mice at E16.5 are similar. Note that Cdk5 mutant muscle cells can form normal AChR clusters in response to agrin. One might ask why AChR cluster sizes in Cdk5 mutant mice are not increased. One explanation is that Cdk5 is not the sole factor in vivo to mediate ACh-induced dispersion. Muscle depolarization and subsequent increase in intracellular calcium could stimulate various kinases including PKC and CaMKII (Schaeffer et al., 2001). It is possible that one of the kinases is involved in regulating AChR clusters in vivo.

What is the destiny of the receptors that are scattered from AChR-rich sites in nonsynaptic areas? Are they recycled to form AChR clusters in the synaptic region? At NMJs in living adult mice, blockade of neurotransmission decreases the half-life of the AChRs and causes their endocytosis (Akaaboune et al., 1999). Dispersed AChRs reaggregate at the junction once neurotransmission is restored. It is unknown whether the dispersed AChRs in developing muscles undergo endocytosis. A key question here is whether such dispersal is a prerequisite to the formation of new clusters. Interestingly, Cdk5 mutant mice, whose dispersing function is impaired, form synaptic AChR clusters similar in size and in number to age-matched controls. These results argue against the idea that the formation of new synaptic clusters requires the dispersion, at least not via the Cdk5-dependent mechanism. The AChR may be provided by local protein synthesis (Schaeffer et al., 2001).

In vertebrates, muscle fibers are innervated by more than one motoneuron during postnatal development. As the NMJ matures, extra inputs are eliminated so that a muscle fiber receives input from a single axon. This process, called synapse elimination, is competitive in nature and is determined by differential activity of involved synapses. Active synapses become stronger, whereas less active synapses are eventually eliminated (Sanes and Lichtman, 1999). The aneural ACh-rich sites in nonsynaptic areas are less active relative to synaptic AChR clusters, creating a difference in activity. Thus, nonsynaptic AChR-rich sites are dispersed upon formation of new AChR clusters. Note that the AChR-rich sites are composed of embryonic AChRs, different from postnatal ones in composition and electric property. Two questions are thus presented: first, whether the two types of AChRs are different in sensitivity toward aggregating and/or dispersing signals, and second, whether their unique firing patterns have different effects on AChR cluster stability. Nevertheless, there is a strong rationale to analyze synapse elimination in Cdk5 mutant mice and to see whether Cdk5 is a punishment signal from active synapses to eliminate neighboring less active synapses at postnatal stages.

Remarkably, mechanisms governing the induction and maintenance of synaptic AChR clusters are similar to those for AChR synthesis. Muscle fibers are multinucleated cells. Typically, a muscle fiber contains 500 to 1000 nuclei, yet only those (about a dozen) beneath the postsynaptic membrane are actively transcribing genes encoding synaptic proteins (Fischbach and Rosen, 1997; Schaeffer et al., 2001). In this model, ACh, also via muscle activation by AChR, suppresses transcription in the entire muscle fiber, whereas transcription in synaptic nuclei is stimulated by positive signals from motoneurons such as neuregulin. Interestingly, however, synapse-specific gene expression appears to be normal in ChAT mutant mice. AChR subunit mRNAs are

distributed in a defined, albeit broader, band that is colocalized with AChR (Brandon et al., 2003; Lin et al., 2005; Misgeld et al., 2002). This is unexpected because denervation of adult muscles leads to overexpression of AChRs in entire muscle fibers (Schaeffer et al., 2001). These results could suggest that nerves may release an ACh-independent signal to suppress AChR expression in nonsynaptic areas. Alternatively, AChR gene expression in adult animals may be regulated by a mechanism more sensitive to activity or electric patterns generated by adult AChRs. Nevertheless, AChR expression and clusters are both regulated by positive and negative signals from the nerve. Negative signals are propagated in the muscle to shut down operation in distance, whereas positive factors reverse the inhibitory effect in the synaptic region (Figure 1). The beautiful coordination between nerves and muscles ensures concentration of AChRs at nowhere but synapses.

In sum, the data presented by Lin et al., together with their earlier studies and those by Sanes and colleagues (Brandon et al., 2003; Misgeld et al., 2002), firmly establish a role of AChR in receptor clustering. It allows us to discuss a model that may be generally applicable to other synapses. Neuronal activity regulates the number of neurotransmitter receptors at synapses including AMPA receptors, NMDA receptors, and GABA receptors. Cdk5 is expressed abundantly in the brain and is implicated in neuron migration, neurotransmission, and neuronal cell death (Cheng and Ip, 2003; Cruz and Tsai, 2004). The findings of this paper suggest that the model and perhaps some of its molecular details such as Cdk5 may be applicable to synaptogenesis in the brain.

Wen C. Xiong and Lin Mei
Program of Developmental Neurobiology
Institute of Molecular Medicine and Genetics
Department of Neurology
Medical College of Georgia
Augusta, Georgia 30912

Selected Reading

Akaaboune, M., Culican, S.M., Turney, S.G., and Lichtman, J.W. (1999). Science *286*, 503–507.

Brandon, E.P., Lin, W., D'Amour, K.A., Pizzo, D.P., Dominguez, B., Sugiura, Y., Thode, S., Ko, C.P., Thal, L.J., Gage, F.H., and Lee, K.F. (2003). J. Neurosci. *23*, 539–549.

Cheng, K., and Ip, N.Y. (2003). Neurosignals *12*, 180–190.

Cruz, J.C., and Tsai, L.H. (2004). Curr. Opin. Neurobiol. *14*, 390–394.

Finn, A.J., Feng, G., and Pendergast, A.M. (2003). Nat. Neurosci. *6*, 717–723.

Fischbach, G.D., and Rosen, K.M. (1997). Annu. Rev. Neruosci. *20*, 429–458.

Fu, A.K., Fu, W.Y., Cheung, J., Tsim, K.W., Ip, F.C., Wang, J.H., and Ip, N.Y. (2001). Nat. Neurosci. *4*, 374–381.

Lin, W., Burgess, R.W., Dominguez, B., Pfaff, S.L., Sanes, J.R., and Lee, K.F. (2001). Nature *410*, 1057–1064.

Lin, W., Dominguez, B., Yang, J., Aryal, P., Brandon, E.P., Gage, F.H., and Lee, K.-F. (2005). Neuron *46*, this issue, 569–579.

Luo, Z., Wang, Q., Zhou, J., Wang, J., Liu, M., He, X., Wynshaw-Boris, A., Xiong, W., Lu, B., and Mei, L. (2002). Neuron *35*, 489–505.

Misgeld, T., Burgess, R.W., Lewis, R.M., Cunningham, J.M., Lichtman, J.W., and Sanes, J.R. (2002). Neuron *36*, 635–648.

Sanes, J.R., and Lichtman, J.W. (1999). Annu. Rev. Neurosci. *22*, 389–442.

Schaeffer, L., de Kerchove d'Exaerde, A., and Changeux, J.P. (2001). Neuron *31*, 15–22.

Trinidad, J.C., and Cohen, J.B. (2004). J. Biol. Chem. *279*, 31622–31628.

Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T.M., Birchmeier, C., and Burden, S.J. (2001). Neuron *30*, 399–410.

DOI 10.1016/j.neuron.2005.05.008